

Contributions of Myristylation to Calcineurin Structure/Function*

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Calcineurin is a serine/threonine protein phosphatase composed of a catalytic subunit, calcineurin A (58 kDa), and a NH₂-terminal myristoylated regulatory subunit, calcineurin B (19 kDa). In order to study the effect of myristylation on calcineurin structure/function, a dual plasmid transfection system was used to generate myristoylated and nonmyristoylated calcineurin B. Both metabolic labeling of calcineurin B with radiolabeled myristic acid and electrospray mass spectral analysis confirmed that myristic acid was covalently and stoichiometrically linked to calcineurin B. Myristoyl and non-myristoyl calcineurin B were reconstituted with recombinant calcineurin A to form native-like heterodimers, and the properties of the two calcineurin forms were examined. Myristylation had no effect on enzymatic activity, calcineurin-immunosuppressant/immunophilin interactions, or Ca²⁺ binding. Surprisingly, myristylation also had no effect on calcineurin heterodimer association with phospholipid monolayers. Fatty acylation, however, significantly influenced the thermal stability of calcineurin, with an approximate 10 °C increase in *t*_{1/2} observed for myristoyl calcineurin when compared to the non-myristoyl form. Myristylation of calcineurin B therefore appears to provide structural stability to the calcineurin heterodimer.

Calcineurin (CaN)¹ is a heterodimeric Ser/Thr protein phosphatase consisting of a 58-kDa catalytic α-subunit (CaNA) and a "regulatory" 19-kDa β-subunit (CaNB). The B subunit has 4 EF-hand Ca²⁺ binding sites with homology to calmodulin, and its primary sequence is well conserved in higher eukaryotes. The biological roles of CaN are diverse, ranging from regulation of ion channels (1–3), gene transcription (4), and neuronal depression (5). Furthermore, CaN is the *in vivo* target of the immunosuppressant/immunophilin complexes in T cells (4, 6, 7). Inhibition of CaN in T cells leads to decreased production of interleukin-2 and attenuation of the cell-mediated immune response.

The eight amino-terminal residues of CaNB following the initiating methionine residue (GNEASYPL) define a consensus

sequence for myristylation (8), and CaNB is in fact post-translationally modified with a myristic acid residue linked to the protein through an amide bond *in vivo* (9). Myristylation is common to a diverse set of proteins, including α-subunit of G-proteins (10, 11), the tyrosine kinases p60^{src} (12–14) and p58^{ck} (15, 16), nitric oxide synthase (17–19), protein kinase A (20), and retroviral capsid proteins (21). Reports have cited myristylation as important for activity (15, 22), protein-protein interaction (21), localization to membrane surfaces (18, 23), Ca²⁺-binding (24–27), and protein stability (28, 29).

For CaN, the biological role of the myristoyl moiety is not known. One mechanism views myristylation as directing membrane association, and in fact, several investigations have focused on CaN-lipid interactions. Politino and King (30) demonstrated that bovine CaN binds acidic phospholipid vesicles in a Ca²⁺-dependent fashion with potentiation of enzymatic activity toward a phosphopeptide substrate increased by up to 23-fold. Furthermore, these studies indicated that the site of lipid interaction was localized to CaNB (31). Recently, myristoylated and nonmyristoylated CaNB were shown to be equally distributed between cytosolic and membrane subcellular fractions in yeast (32), a distribution also found for CaN in bovine brain (33). In addition to studies on localization, it is known that myristylation is not required for association of the subunits of CaN (34, 35) or the binding of Ca²⁺ to CaNB (36). Furthermore, myristylation of CaNB is not required for several calcineurin-dependent functions in yeast, including α-factor-induced arrest and cation stress (32).

Using an approach outlined by Duronio *et al.* (37), we have produced recombinant forms of CaNB with and without the NH₂-terminal myristoyl group and reconstituted them with CaNA. In this work, we address the influence of myristylation on CaN activity, interaction with the immunosuppressant/immunophilin complexes, membrane association, and thermostability. Of these possible effects, our results indicate that myristylation influences only the thermostability of CaN.

EXPERIMENTAL PROCEDURES

Materials—p-Nitrophenyl phosphate (pNPP), MOPS, and Dowex-50W were all purchased from Sigma. Cyclosporin A was from Sandoz Pharmaceutical (Basel). Both 1-palmitoyl-2-oleoyl-sn-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-sn-3-phosphatidylserine (POPS) were from Avanti Lipids (Birmingham, AL).

Expression of CaNA, CaNB, and Myristoyl CaNB in Escherichia coli—The cDNAs for the α isoform of rat CaNA and rat CaNB were kindly provided by Dr. Brian Perrino and Dr. Tom Soderling (38, 39). Plasmid pBB131 encoding the yeast N-myristoyltransferase gene was a gift from Dr. Jeffrey Gordon (37). Both CaNA and CaNB were overexpressed in *E. coli* strain BL21(DE3) using a T7 promoter system and purified as described previously (34). Myristoyl CaNB was prepared in an identical fashion to CaNB with the exception that the N-myristoyltransferase gene was cotransfected along with the plasmid containing the gene for CaNB (pCNBT775-3) into *E. coli* BL21(DE3). Growth of pCNBT775-3/pBB131/BL21(DE3) was maintained in the presence of kanamycin (100 µg/ml) and ampicillin (100 µg/ml). Since the N-myristoyltransferase construct is under the control of the *lac* operon, both

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‡ The abbreviations used are: CaN, calcineurin heterodimer; M-CaN, myristoylated calcineurin heterodimer; hCyPB, recombinant human cyclophilin B; CsA, cyclosporin A; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; POPC, 1-palmitoyl-2-oleoyl-sn-3-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-sn-3-phosphatidylserine; p-nitrophenyl phosphate (pNPP).

Contributions of Myristylation to Calcineurin

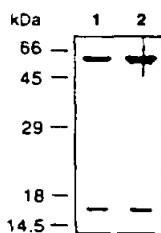


FIG. 1. 13% SDS-PAGE analysis of M-CaN and bovine CaN. *Lane 1*, M-CaN; *lane 2*, bovine CaN. After electrophoresis, the gel was stained with Coomassie Blue to visualize proteins. The molecular masses of protein standards are indicated.

genes were induced by addition of isopropyl-1-thio- β -D-galactopyranoside to 1 mM.

Metabolic Labeling of CaNB with [³H]Myristic Acid—BL21(DE3) cells containing plasmids for rat CaNB and yeast *N*-myristoyltransferase were grown in LB containing 100 μ g/ml each of kanamycin and ampicillin. Cultures were radiolabeled with [³H]myristic acid as described previously (37). Briefly, 5 ml of culture were grown at 37 °C to an A_{600} = 0.5–0.7, induced with isopropyl-1-thio- β -D-galactopyranoside, and incubated for 1 h at 37 °C. Next, 1-ml aliquots were removed into Eppendorf tubes in which 100 μ l of 1 mCi/ml [³H]myristic acid had been previously dried down. Incubation at 37 °C was allowed to continue for an additional 30 min, after which the tubes were placed on ice for 5 min and centrifuged briefly in an Eppendorf centrifuge (2,900 $\times g$). The cell pellets were washed with 1 ml of 0.2 M Tris, pH 8.0 and centrifuged a second time, and the pellets were brought up in a minimal volume of SDS-PAGE sample buffer. The samples were then electrophoresed on a 13% SDS-polyacrylamide gel. After fixing in 30% methanol, 10% acetic acid, the gel was subjected to fluorography with ENHANCE (DuPont). Exposure time to Kodak X-Omat film was 13 h at -70 °C.

Mass Spectrometry of Myristoylated CaNB—Purified myristoylated CaNB was subjected to mass analysis on a Finnigan-MAT electrospray mass spectrometer in the Mass Spectrometry Core Facility at the Mayo Clinic. The sample was prepared by desalting into distilled water, followed by lyophilization. The protein was dissolved in a small amount of 0.1 M acetic acid prior to analysis.

Reconstitution of CaNA with CaNB or Myristoyl CaNB—CaNA was reconstituted with an excess of either CaNB or myristoylated CaNB by combining the partially purified subunits in a molar ratio of approximately 1:6 (CaNA/CaNB), as described previously (34). CaCl₂ was added to a slight excess (1–10 μ M) over EDTA present in the buffers from the previous chromatographic step. The mixture was incubated for at least 4 h at 4 °C, then chromatographed over a calmodulin-Sepharose affinity column. Since calmodulin is known to bind CaNA, only CaN heterodimers are retained on the column while excess CaNB is washed through. The heterodimers were eluted with a buffer containing 0.1 mM EGTA. Fractions containing enzymatically active calcineurin heterodimer were pooled and subjected to S-300 gel filtration chromatography for further purification and selection of CaN heterodimers. Purity of pooled fractions after this step was >95%, as judged by 13% SDS-PAGE gels stained with Coomassie Blue (Fig. 1).

pNPP and ³²P-R-II Peptide Assays—CaN or myristoylated calcineurin (M-CaN) activity was measured by both pNPP (40) and ³²P-R-II peptide assays (41) as described previously. Assays using pNPP as substrate were carried out at 25 °C, while assays using ³²P-R-II peptide

substrate were at 30 °C.

CaN Monolayer-binding Assays—Binding of M-CaN and CaN to lipid monolayers was studied using a Wilhelmy-type film balance (42). The apparatus consisted of a 5-cm diameter Teflon dish containing 19.5 ml of buffer (25 mM Tris, 0.15 M NaCl, 1 mM dithiothreitol, pH 7.5) mounted on a stirplate. A 24-gauge nichrome wire suspended from a Cahn Electrobalance was used for surface pressure measurements (43). For these experiments, the surface pressure was defined as the difference in surface tension between buffer alone and buffer with the lipid film. The dish was enclosed in a plexiglass box which was atmospherically controlled with hydrated argon and a constant temperature of 24.4 °C. The dish was stirred throughout the experiment with a small Teflon coated stir bar at 50 rpm.

After addition of buffer to the dish, excess buffer was removed, and the surface was cleaned by vacuum aspiration. Next, a solution of 80 mol % POPC, 20 mol % POPS in 95% hexane, 5% ethanol (v/v) was spread at the air-buffer interface by dropwise addition to the surface with a Hamilton syringe. The monolayer was equilibrated until the

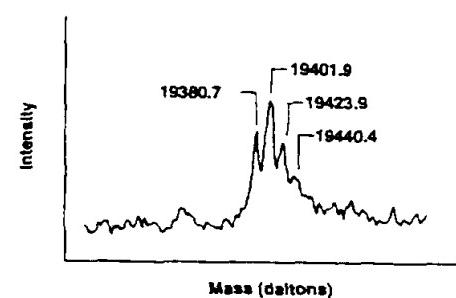


FIG. 2. Positive ion electrospray mass spectrum of myristoyl CaNB (see "Experimental Procedures" for protein preparation). Each peak is labeled with its respective mass in daltons.

change in surface pressure with respect to time was ≤ 0.01 mN m⁻¹ min⁻¹, usually 20–40 min. M-CaN or CaN was injected into the subphase through a port located beneath the monolayer to a final concentration of 0.1 μ M. The increase in surface pressure was then followed as a function of time until no further increases could be detected, on the order of ≤ 30 min. The change in surface pressure was calculated by subtracting the initial surface pressure from the final. Initial surface pressures in the range of 10–30 mN/m were explored. The surface pressure-area diagram for the lipid mixture used was determined as described previously (44).

CaN Thermostability Assays—For assays examining CaN thermostability, a small aliquot of 6 μ M M-CaN or CaN was incubated at various temperatures, ranging from 34–65 °C for 3 min. A 10- μ l aliquot was removed and added to a cuvette containing 500 μ l of assay buffer (25 mM MOPS, 0.1 mM CaCl₂, 1 mM MnCl₂, 1 μ M calmodulin) and 10 mM pNPP (the final concentration of CaN was 120 nM). The increase in absorbance was followed spectrophotometrically at 410 nm for 3 min at 30 °C. The slope of the absorbance change from 0.5 to 2.5 min was used to calculate phosphatase activity. Over this time interval the absorbance change was linear, indicating no appreciable renaturation had occurred.

RESULTS

Overexpression of CaNB and Myristoyl CaNB

Coexpression of rat CaNB with or without the gene encoding the yeast *N*-myristoyltransferase in *E. coli* yielded myristoylated CaNB and CaNB, respectively. Metabolic labeling of the myristoyl form of CaNB using [³H]myristic acid, followed by SDS-PAGE and autoradiography confirmed that CaNB was myristoylated (data not shown). Both myristoyl CaNB and CaNB were purified in the same fashion on DEAE-Sepharose CL-6B, G-75 gel filtration, and Mono-Q columns, and both appeared >90% pure as judged by Coomassie-stained gels (data not shown). Myristoyl CaNB migrated during SDS-PAGE with an apparent molecular mass of 16 kDa, approximately 3 kDa less than the expected mass of 19.4 kDa, but identical to that of the native, myristoylated subunit isolated from bovine brain. To further verify that coexpression of CaNB with *N*-myristoyltransferase led to covalent attachment of myristic acid to CaNB, purified myristoyl CaNB was subjected to electrospray mass spectral analysis (Fig. 2). The experimentally determined molecular mass obtained for myristoyl CaNB (19,380.7) was within 0.01% of the calculated mass (19,380.0) thereby demonstrating that the anomalous mobility of the myristoylated subunit is due solely to myristylation and not additional post-translational modifications or proteolysis. The envelope of peaks in Fig. 2 separated by approximately 22 mass units is diagnostic of the different Na⁺ isoforms of the protein.

Reconstitution of CaNA with CaNB or Myristoyl CaNB

Effect of Myristylation on Enzyme Activity and Inhibition by Cyclosporin A/Cyclophilin B—It has been previously shown that CaNA and CaNB can be reconstituted into an active, native calcineurin-like heterodimer (34). In a similar fashion, myristoyl CaNB can be reconstituted with CaNA and purified

TABLE I
Enzymatic characterization of non-myristoyl and myristoyl calcineurin

Activities of CaN and M-CaN were measured with two substrates, R-II peptide and pNPP as described under "Experimental Procedures." Two independent preparations of recombinant CaN are shown. Data were fit to the Michaelis equation by a nonlinear least squares method. The data presented are the mean \pm S.E. and were determined from three separate trials.

	Substrate			
	R-II Peptide		pNPP	
	K_m	V_{max}	K_m	V_{max}
	μM	$\mu\text{mol min}^{-1}$ mg^{-1}	μM	$\mu\text{mol min}^{-1}$ mg^{-1}
CaN				
Preparation 1	130 \pm 24	12 \pm 2	57 \pm 4	5.6 \pm 1.8
Preparation 2	55 \pm 32	7.7 \pm 1.4	81 \pm 24	11 \pm 1
M-CaN				
Preparation 1	150 \pm 12	18 \pm 2	20 \pm 5	8.7 \pm 0.5
Preparation 2	52 \pm 6	12 \pm 2	20 \pm 3	11 \pm 3

to homogeneity (Fig. 1). Using two independent preparations of purified CaN and M-CaN, the effects of myristoylation on recombinant calcineurin activity were examined using both pNPP and R-II peptide as substrates. The data do not indicate a significant difference in phosphatase activity for CaN versus M-CaN (Table I). For both substrates tested, the kinetic constants for M-CaN and CaN are comparable. Myristoylation, therefore, appears to have no significant effect on the phosphatase activity of calcineurin using these two substrates.

The effect of myristoylation on the interaction of CaN with immunosuppressant/immunophilin complexes was tested by assessing the inhibition of CaN and M-CaN by cyclosporin A/recombinant human cyclophilin B (CsA/hCyPB). Using ^{32}P -R-II peptide, the dose-response curves for M-CaN versus CaN were identical (Fig. 3). For both myristoyl and non-myristoyl calcineurin, 50% inhibition was observed at $\sim 20 \text{ nM}$, the same concentration observed for native CaN (40). The results indicate that myristoylation has no effect on the binding of CsA/hCyPB to CaN.

Effect of Myristoylation on the Interaction of Calcineurin with Phospholipids—Calcineurin has been previously shown to associate with lipid surfaces *in vitro* and *in vivo* (30–32, 45, 46). To assess the degree to which myristoylation contributes to lipid binding we measured the change in surface pressure induced by CaN binding to POPC/POPS monolayers. The lipid film was in the liquid expanded state and exhibited no phase transitions in the surface pressure range between 0.1 mN/m and monolayer collapse (data not shown). For both CaN and M-CaN, a protein concentration of 0.1 μM was sufficient to saturate the surface pressure changes observed (data not shown). Fig. 4 shows a plot of the change in pressure as a function of the initial surface pressure in the range of 10–30 mN/m initial surface pressure. The x -intercept of such plots represents the maximum initial surface pressure of the monolayer at which the interaction of protein and lipid is energetically favorable for protein insertion. For bovine CaN, CaN, and M-CaN, the linear fits of the data are similar, with x -intercepts near 25 mN/m. Thus, there appears to be no substantial difference in interaction between bovine CaN, CaN, or M-CaN with the phospholipid membrane.

Effect of Myristoylation on Thermal Stability—For cAMP-dependent protein kinase, myristoylation was shown to stabilize the protein when subjected to thermal denaturation (28). It was therefore postulated that myristoylation may serve a similar role in CaN. M-CaN or CaN were each preincubated at various temperatures, after which the phosphatase activity toward pNPP was determined. As shown in Fig. 5, myristoyla-

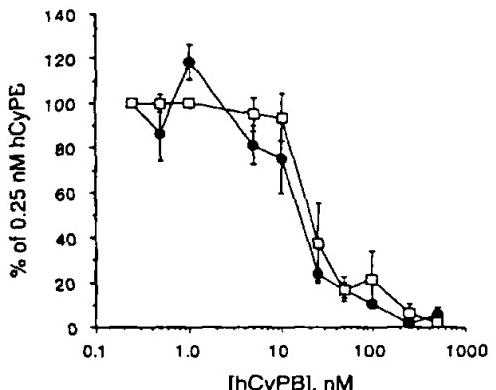


FIG. 3. Inhibition of CaN (□) and M-CaN (●) by cyclosporin A/cyclophilin B (hCyPB) as a function of hCyPB concentration using the R-II peptide as substrate. The concentration of cyclosporin was held constant at a saturating level (1 μM) with respect to cyclophilin. The data presented represent the mean of three trials \pm S.E.

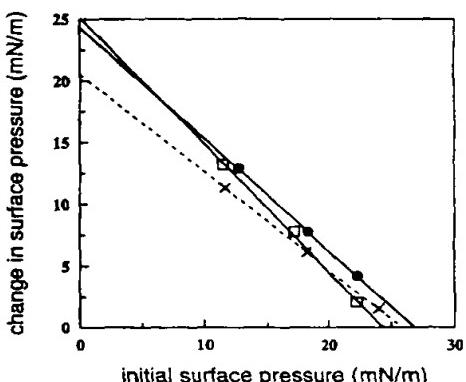


FIG. 4. Effect of myristoylation on CaN interaction with lipid monolayers. Bovine CaN, CaN, and M-CaN were examined for their ability to bind to phospholipid monolayers consisting of 80% POPC, 20% POPS overlying buffer (25 mM Tris, 0.15 M NaCl, 1 mM dithiothreitol, pH 7.5) as described under "Experimental Procedures." The change in surface pressure induced by protein binding was plotted as a function of the initial surface pressure. For all measurements, the final protein concentration was 0.1 μM . A least squares fit was applied to the data for bovine CaN (x), CaN (□), and M-CaN (●).

tion provides an additional 12 °C stabilization to thermal inactivation. The temperatures at which half-maximal activity was observed ($t_{1/2}$) for CaNA/B and CaNA/M-B are 50 and 62 °C, respectively.

DISCUSSION

Previous studies have determined that NH₂-terminal myristoylation can influence a diverse set of protein functions including activity, protein-protein interactions, membrane localization, Ca²⁺ binding, and protein stability. However, the role of fatty acylation to calcineurin has not yet been fully explored. Given the ability to prepare calcineurin with and without the myristoyl moiety, a direct comparison of the biochemical properties of these two proteins has yielded insight into the role of myristoylation.

The enzymatic activity of CaN was unaffected by myristoylation. Both recombinant myristoyl and non-myristoyl calcineurin had comparable kinetic constants and were both similar to native bovine CaN as determined using two different substrates, pNPP and R-II peptide. This result is in contrast to a report citing myristoylation as required for CaN activity (22). However, a recent finding that myristoyl and non-myristoyl

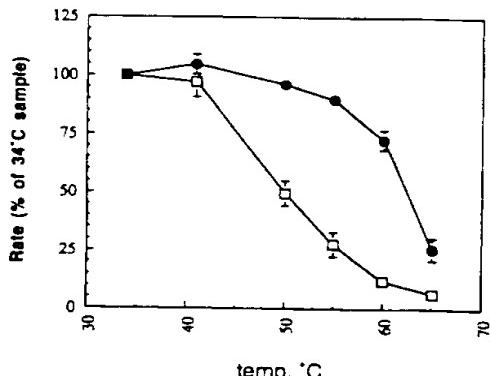


FIG. 5. Effect of elevated temperature on CaN and M-CaN activity. An aliquot of CaN (□) or M-CaN (●) was preheated at various temperatures (ordinate), then assayed for activity using pNPP as substrate in buffer containing 25 mM MOPS, 1 mM MnCl₂, 0.1 mM CaCl₂, pH 7.0, at 30 °C. The abscissa represents activity as a percent of the activity at 34 °C. For both proteins, the data presented are the mean of three experiments ± S.E.

calcineurin were functional in a calcineurin-dependent yeast strain (32) also indicates that myristoylation is not required for enzyme activity.

Calcineurin-immunosuppressant/immunophilin interactions were also unaffected by calcineurin myristoylation. Both myristoyl and non-myristoyl forms of calcineurin had identical inhibition profiles when treated with CsA/hCyPB. At least for this drug/immunophilin complex, myristoylation does not influence binding to CaN. This result is consistent with recent structural data for CaN complexed with FK506/FKBP which shows that the myristoyl group is positioned far from the CaN/immunosuppressant interface (47). In addition to crystallographic evidence, an *in vivo* study of the effect of myristoylation on drug/immunophilin-calcineurin interactions revealed no differences between myristoyl and non-myristoyl protein (32).

The majority of investigations on the role of protein myristoylation have focused on the membrane-binding properties of the parent protein. Localization of an enzyme to the inner leaflet of the plasmalemma may increase the overall rate of reaction and bring signaling molecules to their proper location for signal transduction. In accord with these observations, potentiation of CaN activity toward phosphorylated protein substrates is observed in the presence of negatively charged phospholipids (30). In the case of recoverin, a retinal rod protein, membrane association is controlled by Ca²⁺ binding. This "myristoyl-switch" is believed to be the regulatory mechanism controlling recoverin function (24, 25).

In contrast to studies where myristoylation was found to be a determinant of localization, we conclude that myristoylation does not influence binding to phospholipid membranes as evidenced by comparable binding properties of M-CaN and CaN to phospholipid monolayers. Both forms of CaN insert into the monolayer, resulting in a positive change in surface pressure. Interestingly, the surface pressures at which the various forms of calcineurin bind (≤ 26 dynes) are below those accepted for biological bilayers, ranging from 30 to 35 dynes (48–50) to as high as 50 dynes (51, 52). However, the difference between our measurements and the accepted range may reflect nonphysiologic conditions, phospholipid specificity, or the need for additional biologic components in the monolayer (protein, cholesterol, diacylglycerol) and/or medium.

The fact that myristoylation does not appear to be a contributing factor to membrane association is in agreement with previous qualitative fractionation experiments using myristoyl and non-myristoyl CaNB (32) and vesicle binding experiments

using myristoyl and non-myristoyl peptides (53). As shown by Peitzsch and McLaughlin (54), the myristoyl moiety contributes only 8 kcal/mol association energy ($K = 10^4 \text{ M}^{-1}$) in model peptide experiments. For pp60^{WT}, a string of basic residues at the amino terminus was shown to contribute significantly to protein localization, accounting for the significantly higher affinity of pp60^{WT} for membranes ($K = 10^7 \text{ M}^{-1}$) (55, 56). Together, these observations reinforce the emerging dogma that myristoylation is not the sole determining factor for protein association with lipid surfaces. In the case of calcineurin, basic residues are notably absent from the first 20 amino acids of the amino terminus, in contrast to pp60^{WT}. A polybasic domain does exist, however, in the amino-terminal region and it is conserved in both mammals and yeast. The contributions of this domain to membrane binding await further studies. However, given the surface pressure measurements observed here, a portion of CaN may actually penetrate the membrane surface.

Previous studies on CaN localization using yeast fractions (32) and sonicated lipid (30) are somewhat limited by the characterization of the membranes used and should be taken with caution in the context of physiologic relevance. Mechanical disruption of the cellular membrane produces extremely heterogeneous lipid dispersions that may have properties unlike the cellular form from which they were derived. Moreover, sonication of synthetic lipid mixtures as utilized in the preparation of vesicles for the bovine CaN study results primarily in a dispersion of small unilamellar vesicles, which may yield artifactual results in protein-membrane interaction studies. Amphipathic proteins, such as cytochrome b_5 , have been shown to bind to larger vesicles less avidly than to smaller ones (57). This difference in partition coefficient is thought to be a result of the higher curvature/lower surface pressure of smaller vesicles. A similar dependence of membrane surface pressure on insertion of fatty acids into monolayers has also been observed (58). Thus, one must carefully consider the physical state of any model lipid surface used before drawing conclusions as to whether or not protein-membrane interactions are biologically relevant.

It is clear that myristoylation of CaNB conveys thermal stability to CaN. For cAMP-dependent protein kinase, myristoylation was shown to be a determinant of thermal stability (28, 29). In a similar fashion, CaN is also stabilized by myristoylation as evidenced by the maintenance of activity of myristoylated calcineurin compared to non-myristoyl after exposure to elevated temperatures. A 12 °C difference in $t_{1/2}$ between the two forms indicates the conformation assumed by myristoylated CaN is significantly more stable. Examination of x-ray crystal structure images of the myristoyl region on CaNB show that the myristoyl group occupies a hydrophobic cleft, partially shielding several residues from contact with the aqueous environment (47). The myristate appears to be anchored via multiple hydrophobic contacts and may contribute to the overall structure of the enzyme in a manner similar to that postulated for cAMP-dependent protein kinase (29). The implications of this finding are not immediately apparent, but may involve the adaptability of the host organism to heat stress or folding of CaNB.

Although we have defined a role for myristoylation in stabilizing CaN structure, there are certainly other functions not yet examined that may be influenced by myristoylation or be myristoyl-dependent. Additional experiments examining the parameters governing membrane association and stability of calcineurin should help clarify the role of CaN myristoylation.

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